***In situ* grazing experiments apply new technology to gain insights into deep-sea microbial food webs**

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ABSTRACT

Predation by grazing protists in aquatic habitats can influence prokaryotic community structure and provides a source of new, labile organic matter. Due to methodological difficulties associated with studies of deep-sea (below photic zone) microbiota, trophic interactions between eukaryotes and prokaryotes in mesopelagic and bathypelagic realms are largely obscured. Further complicating matters, examinations of trophic interactions using water samples that have been exposed to upwards of hundreds of atmospheres of pressure change prior to initiating experiments can potentially introduce significant artifacts. Here we present results of the first study of protistan grazing in water layers ranging from the euphotic zone to the bathypelagic, utilizing the Microbial Sampler-Submersible Incubation Device (MS-SID)^{*} that makes possible *in situ* studies of microbial activities. Protistan grazing in the mesopelagic and bathypelagic realm of the East Mediterranean Sea was quantified using fluorescently labeled prokaryotes (FLP) prepared from the naturally-occurring prokaryotic assemblages. These studies reveal daily prokaryotic removal due to grazing ranging from $31.3 \pm 5.9\%$ at 40 m depth to $0.5 \pm 0.3\%$ at 950 m. At 3540m depth, where a chemocline habitat exists with abundant and active prokaryotes above Urania basin, the daily consumption of prokaryotes by protists was $19.9 \pm 6.6\%$ of the *in situ* abundance.

Running title: Protistan phagotrophy in deep-sea

^{*} Abbreviations

MS-SID: Microbial Sampler-Submersible Incubation Device

FLP: Fluorescently labeled prokaryotes

1. INTRODUCTION

Protists are unicellular eukaryotes that interact in several ways with bacterial and archaeal populations, shaping substantially the structure of these marine communities. Phagotrophy is an ancient mode of nutrition for protists that predates photosynthesis (Cavalier-Smith, 2002 and references therein), resulting in widespread distribution of this mode of nutrition among the major phyla in the protistan tree of life. Phagotrophic grazing can affect the quantity, activity, and physiological state of prey organisms, and sometimes protists exhibit prey preferences (Jezbera *et al.*, 2006; Jürgens and Massana, 2008 and reference there in; e.g. Simek *et al.*, 1997). Indeed, protistan assemblages may graze on the order of 25 to > 100% of the measured daily production of prokaryote plankton (Sherr and Sherr, 1994), resulting in the consumption of prokaryote biomass at approximately the same rate as it is produced (Pernthaler, 2005). Through grazing, protists actively participate in the transfer of organic carbon from the dissolved fraction to higher trophic levels, in a heterotrophic food chain involving bacteria, nanoflagellates (2-20µm), and ciliates, that has been termed the “microbial loop” (Azam *et al.*, 1985). Protists are capable of inducing significant shifts in prokaryote community composition, of enhancing microdiversification, and of impacting prokaryote community evenness (Simek *et al.*, 1997). Dominant groups and size classes of common protistan grazers (particularly small flagellated protists and ciliates) as well as some known ecological impacts of grazing were recently summarized (Jürgens and Massana, 2008; Pernthaler, 2005). Through their grazing activities, protists are known to regenerate nutrients and modify or re-mineralize organic matter (e.g. Jumars *et al.*, 1989; Sherr and Sherr, 2002).

In recognition of their importance in marine aquatic communities, protozoa are considered in numerical models of carbon cycling and in paradigms of surface and deep-ocean microbial ecology (Aristegui *et al.*, 2009). Indeed, as phagotrophic protists and viruses are the

main sources of mortality for marine microbes (Arístegui *et al.*, 2009; Fuhrman and Noble, 1995), they are presumed to be important in the cycling of both particulate and dissolved organic matter, however their specific roles in carbon cycling are not well understood. A large portion of oceanic primary production is usually respired in the well-oxygenated mixed layer through grazing and microbial remineralization processes, and returned to the atmosphere as CO₂. Microbial processing of dissolved and particulate organic matter in the deep sea remains poorly constrained, particularly regarding the impact of protist grazing on prokaryotic communities, which aside from shaping prokaryotic communities at depth, could serve as a fresh source of labile organic matter in deep ocean.

With increasing exploration of deeper ocean mesopelagic (200-1000m) and bathypelagic (1000-4000m) realms, we now know that while overall microbial cell counts tend to decline with depth, protists not only are present, but their communities in the deep ocean, including Gulf of Mexico are diverse (Lopez-Garcia *et al.*, 2001; Sauvadet *et al.*, 2010; Schnetzer *et al.*, 2011). Furthermore, where oxyclines and redoxclines exist, protist numbers and diversity increase relative to the adjacent deep-ocean waters (Edgcomb *et al.*, 2011b; Stock *et al.*, 2012). Studies of activities of phagotrophic protists have been conducted mainly within the photic zone, using samples from up to 200m depth (e.g. Christaki *et al.*, 1999; Karayanni *et al.*, 2008; Tsai *et al.*, 2012). Little is known about the grazing impact of protists on prokaryotic communities below the upper mesopelagic zone and in other “challenging” environments such as hydrocarbon polluted sea water in the Gulf of Mexico, where grazing protozoa might be able to encourage re-mineralization of growth-limiting nutrients, and where predation can possibly result in enhanced prokaryotic growth and activity, fostering the utilization of hydrocarbons as a carbon source for the microbial communities (Beaudoin *et al.* this issue).

Grazing studies to date have relied upon hydrocasting operations for collection of water samples from the water column typically via Niskin-bottle hydrocast operations. These samples are brought to ship's deck, and then typically drawn from the Niskin bottle for subsequent studies. While ship-board grazing studies of near surface samples collected with Niskin bottles likely introduce few artifacts associated with the collection and transfer of ocean samples into experimental containers, similar handling of deeper samples may result in dramatic alteration of microbe physiological state, particularly when studying anoxic and/or deep-water column environments (Feike *et al.*, 2012). Such perturbations can affect downstream studies of various microbial activities, including protist grazing. Many microbial eukaryotes are extremely sensitive to physico-chemical changes, particularly oxidation-reduction (redox) state, significant sudden pressure shifts, and possible temperature changes. Consequently, Niskin sampling may only recover a fraction of the total *in situ* eukaryotic community from anoxic waters or redox interfaces below ~250m. This phenomenon was observed in water samples from oxycline (300m depth) and 900m deep anoxic water samples from the Cariaco Basin, Venezuela (Edgcomb *et al.*, 2011a). The same may be true from oxic deep mesopelagic and bathypelagic waters, where pressure changes during sample retrieval can be significant. Regardless of the degree to which individual water samples from different sites and depths are affected by traditional Niskin bottle retrieval, it makes sense that for a comprehensive picture of *in situ* microbial community activity the best strategy is to conduct rate process measurements *in situ* in order to minimize potential artifacts associated with experimental methods and to interpret the data in the context of the local environment. For complete examinations of hydrocarbon impacts on mesopelagic and bathypelagic microbial communities in locations such as the Gulf of Mexico, it is important to include studies of microbial eukaryotes, as they participate in marine nutrient cycles both directly, and indirectly through predation on Bacteria and Archaea. For assessing carbon

turnover due to phagotrophy, technology is required that minimizes the artifacts associated with sample handling by facilitating *in situ* incubation studies.

Here we present the first *in situ* study of phagotrophic protist grazing activities using water samples collected and incubated at selected depths down to 3000m in the Eastern Mediterranean Sea, and also one experiment performed in a deep-sea redoxcline at 3540m. Normally, with shipboard incubations, each experiment for each water depth studied, would be replicated. Given the 4 - 16 hours of wire time required for each study, it was not possible to replicate experiments. As a result, ecological interpretations of data presented here must be interpreted cautiously, and require future replication for depths of interest. Results of this study of protistan phagotrophy, including in the bathypelagic realm, provide a first demonstration of the use of *in situ* approaches for providing insights into the activities of deep ocean protists. The grazing impact of the protistan community was estimated by (i) short term grazing experiments, tracing the ingested fluorescently labeled prokaryotes (FLPs), as described in Sherr et al. (1987) and (ii) long term incubations, estimating the rate of disappearance of the FLPs, according to Vazquez-Dominguez et al. (1999).

2. MATERIALS AND METHODS

2.1 Site description

Mediterranean Sea is characterized by an eastward progression of increasingly oligotrophic conditions, and the Eastern basin is defined as an ultra-oligotrophic, phosphorus-depleted system (Sarmiento *et al.*, 1988; Thingstad *et al.*, 2005). The study was conducted in Libyan Sea, Eastern Mediterranean (35° 13.840'N, 21° 28.478'E) 200 km west of Crete on the Mediterranean Ridge (Figure 1), at the boundary of the accretionary complex and the so-called Inner Plateau of the ridge. At this location lies the Urania Basin, a sea floor depression that is

filled with anoxic and highly saline brine that is characterized by the highest concentration of sulfide ever encountered in the marine environment (12–20mM). The Urania hypersaline basin, as all Mediterranean deep Hypersaline anoxic basins (DHABs), is separated from the water column by a halocline possessing a steep salt gradient (3.9% to 27% [w/w] salinity) between anoxic hypersaline brine and overlying seawater with high bacterial activity (Yakimov *et al.*, 2007a). The data presented in this study derive from 3 oceanographic expeditions carried out in 2011 and 2012 aboard the R/V *Urania* of the Italian National Research Council (CNR) and in 2011 on the R/V *Atlantis* of the Woods Hole Oceanographic Institution (WHOI).

2.2 Preparation of the prey analogues, FLP

Water samples were collected from the Eastern Mediterranean Sea at 3000m in September 2011. After pre-filtering through 0.8µm pore size filters to exclude protists and larger eukaryotes, prokaryotic cells were concentrated by tangential flow filtration (Pellicon System, Millipore Co. equipped with a Biomax 500K polyethersulfone membrane). The concentrated sample was used as inoculum into sterile seawater to which 0.1% Luria Broth (LB) medium was added and the enrichment cultures were kept at 12°C. When they attained exponential growth (as determined by microscopy counts), the cells were pelleted by centrifugation (20min at 2000g), resuspended into sterile seawater and grown at the same temperature for an additional time that ranged from 5 to 15 days. Prokaryotic cells from the enrichments were harvested and stained (as described below) when the size of the prey analogues was within the size range of the natural prokaryotic population along the depth transect. FLPs from enrichments were prepared for the grazing incubations conducted at 40, 200, 500, 950 and 3000m.

For grazing studies conducted in the Urania interface (halocline), prey analogues were generated by prefiltering 80L of halocline water collected in Niskin bottles, using 1.2µm pore

size filters, and then concentrating the filtrate using tangential flow filtration as described above. In this manner, the concentrated indigenous prokaryotic population was used directly for staining, as described below.

The cells were stained as described in Sherr et al. (1987) with minor modifications. The prokaryotic enrichments (and the indigenous concentrated population for Urania Interface and the prokaryotic enrichments for the different depths) were centrifuged at 14000g for 12min and the pelletized cells were re-suspended in $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (pH: 9.5). The dye 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) was added at a final concentration of 0.8mg mL^{-1} , and the mixture was incubated at 60°C for 3h (vortex mixed every 15min). Staining was followed by three washing steps, using $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (pH: 9.5) to remove excess DTAF. Enumeration of prey analogues was performed microscopically. The microscopic observation of the stained cells revealed good staining intensity and absence of cell clumps. They were stored at -20°C and thawed on ice before use.

2.3 Use of the MS-SID.

C. Taylor and McLane Research Laboratories developed automated micro-laboratories for conducting multiple tracer incubation studies during cabled or free-drifting deployments (Taylor *et al.*, 1993; Taylor and Doherty, 1990). This technology was recently modified by C. Taylor, V. Edgcomb, and McLane Research Laboratories into an instrument (Figure 2A) that conducts tracer incubations in combination with in situ microbial sampling and preservation.

The modular Microbial Sampler-Submersible Incubation Device (MS-SID) (Figure 2A) consists of a 2L syringe-like incubation chamber, a 50-port Fluidic Distribution Valve, a micro-annular gear pump for microbial sampling, a tracer injector, 48 Fixation Filter Units (FFUs, inset) for collection & in situ preservation, High Range Conductivity, Temperature, Density (CTD)

sensors (Neil Brown Ocean Sensors, Incorporation, Falmouth, Massachusetts (MA), United States of America (USA)), Aanderaa oxygen optode (Aanderaa Data Instruments, Incorporation, Attleboro, MA, USA), 2 NTURTD 0-124 turbidity sensors (WET Labs, Incorporation, Philomath, Oregon, USA), and real-time bi-directional communication, and electronic control. The MS-SID possesses a Synchronous Digital Subscriber Line (SDSL) data link that multiplexes digital signals from up to 5 sensors at once, and bi-directionally transmits signals to and from the instrument for triggering “adaptive sampling” operations via ordinary conducting hydro-wire. This allowed us to precisely position the instrument at the target depth in the water column and then trigger sampling for our grazing studies. This was particularly important for the precise collection of Urania halocline samples, since the halocline was less than 2 m thick.

2.4 Grazing experiments

For the euphotic zone (40m) and the mesopelagic realm (200m, 500m and 950m) short-term grazing experiments were conducted in November 2011. For the bathypelagic realm (3000m and Urania Interface-3540m) both long- and short-term grazing experiments were conducted in October 2012.

2.4.1 Short grazing experiments

For the short term experiments the FLPs-water mixtures were immediately subsampled at time zero (T_0) and after 5, 10, 15, 20 and 40min. At each of these time points, 250mL subsamples were sent by the MS-SID from the incubation chamber into Tedlar® bags containing formaldehyde (for final concentration of ~2.5%). After retrieval of the instrument, protists in the fixed subsamples were collected by filtration onto 0.8µm polycarbonate filters (for estimations of the total eukaryotic abundance and enumeration of the number of ingested FLPs per eukaryote). For the T_0 samples, 0.2 µm polycarbonate filters were used for estimating the initial prokaryotic and FLPs abundances. The FLPs ingestion rate (FLPs consumed grazer⁻¹ min⁻¹) was calculated by

the slope of the regression line when the number of FLPs consumed per grazer was plotted against time (Sherr and Sherr, 1993). The **clearance rate** ($\text{nL grazer}^{-1} \text{h}^{-1}$) was calculated by dividing the FLPs ingestion rate by the FLPs concentration. The clearance rate of each protistan group was multiplied by the natural prokaryotic abundance to estimate the specific (for each protistan group) **grazing rate** ($\text{prokaryotes grazer}^{-1} \text{h}^{-1}$). The **grazing effect** ($\text{prokaryotes mL}^{-1} \text{h}^{-1}$) of each group on the natural assemblage of prokaryotes was calculated by multiplying the group-specific grazing rate by the relative abundance of each group and the cumulative grazing effect of the total grazing community was determined by adding the grazing effects of each group. Prokaryotic turnover rates ($\% \text{d}^{-1}$) were also calculated and expressed as the percentage of the prokaryotes consumed daily ($\text{prokaryotes mL}^{-1} \text{d}^{-1}$) out of the standing prokaryotic abundance.

2.4.2 Long-term experiments

For the long-term experiments, total bacterivory was estimated by following the FLPs disappearance method (Vazquez-Dominguez *et al.*, 1999). The 2L FLPs-seawater mixtures were subsampled in replicates at T_0 and 12h after the addition of the prey analogues. After retrieval of the instrument, the fixed subsamples ($\sim 400\text{mL}$) were collected from the Tedlar® bags and 1-50mL were filtered onto $0.2\mu\text{m}$ polycarbonate filters (for estimations of the total prokaryotic and FLPs abundances at the beginning and at the end of the experiments), and 60-260mL were filtered onto $0.8\mu\text{m}$ filters for enumeration of eukaryotic cells. The equations from Salat and Marasse (1994) were used to calculate the grazing effect ($\text{prokaryotes mL}^{-1} \text{h}^{-1}$) of the total protistan community. The rest of the estimators were calculated using the equations described above.

2.5 Epifluorescence Microscopy:

Total eukaryotic and prokaryotic populations for all depths, where grazing studies were conducted, were enumerated using epifluorescence microscopy (Porter and Feig, 1980). A portion of each filter (ranging from one quarter to the whole filter) was stained with DAPI (1 μ g mL⁻¹). The stained filters were mounted with immersion oil onto glass slides and observed with a Zeiss Axio Imager M.2 epifluorescence microscope at 1000x magnification. Microscopic eukaryotes and prokaryotes were counted using DAPI filter set (excitation: G 365, emission: LP 420, beamsplitter: FT 580). For the short-term experiments, observed eukaryotes were categorized (based on their DAPI-stained image) into flagellates and ciliates. Furthermore, flagellates were grouped by the presence or absence of chlorophyll autofluorescence using the filter set 15 (excitation: BP 546/12, emission: LP 590, beamsplitter: FT 580) in pigmented flagellates (PF) and (non-pigmented) heterotrophic flagellates (HF). The size of all flagellates observed from both groups was <20 μ m so the final categories were established as pigmented nanoflagellates (PNF) and heterotrophic nanoflagellates (HNF), accordingly. For each sample, at least 250 protists in more than 80 fields were inspected for ingested FLPs using the FITC filter set at each time point and replicate count. Three replicate counts (technical replicates) were performed per depth and time point. The accuracy of the DAPI counts for eukaryotes was validated with fluorescence in situ hybridization (FISH) using the general eukaryotic probe Euk1209 (Giovannoni *et al.*, 1988) and method according to Pernthaler *et al.* (2001).

3. RESULTS

The vertical profiles of temperature, oxygen concentration and salinity of the water column above Urania Basin down into the halocline of Urania Basin in the East Mediterranean Sea are shown in Figure 3A. A steep thermocline from the surface to 150m was followed by a slower decrease in temperature until ~1000m depth. Temperature remained stable at 14.2 $\pm$ 0.2°C

from 1000m to ~3540m, the depth of the halocline above Urania basin. When reaching the Urania interface (Figure 3B) a sharp increase in salinity from 3.78 to ~12.0‰ occurred across this ~1.5m halocline, with a simultaneous decrease in oxygen concentration (from $192.0 \pm 0.3 \mu\text{M}$ to undetectable).

3.1 In situ abundance of prokaryotic and protist populations

A decline in abundances of prokaryotes and protists with depth from the euphotic zone to the mesopelagic realm was observed (Figure 4). For the lower mesopelagic zone (500m and 950m), our data showed that prokaryotic abundance remained stable ($5.41 \pm 1.02 \times 10^4 \text{ cells mL}^{-1}$ and $5.64 \pm 0.89 \times 10^4 \text{ cells mL}^{-1}$ accordingly), whereas the concentration of protists continued to decline from $29.7 \pm 8.3 \text{ cells mL}^{-1}$ to $15.3 \pm 9.2 \text{ cells mL}^{-1}$. Ciliates were the least abundant protistan group observed during the study period and ciliates were almost undetectable at depths below 200m. The inability to estimate ciliate abundances below ~200m depth is due to a combination of low in situ abundance and the size of subsamples collected (due to technical limitations the sample volume used for counting was up to 200mL). At 3000m the average cell counts for prokaryotes was $8.89 \pm 0.58 \times 10^4$ per mL and for total protists 223 ± 15 per mL, while in Urania Interface those values reached $1.64 \pm 0.22 \times 10^6 \text{ cells mL}^{-1}$ and $1071 \pm 117 \text{ cells mL}^{-1}$, respectively.

3.2 Estimators of phagotrophy

Estimators of phagotrophy for all water depths examined in this study are presented in Table 1 and Figure 5. The average values for prokaryotic turnover rate per day declined from 31.3±5.9 % of cell abundance at 40m depth to 8.5±2.6% at 200m, 0.8±0.5% at 500m, and 0.5±0.3% at 950m. For the deepest, most challenging, water layers (3000m and Urania interface at 3540 m), we applied both the short- and long-term approaches in order to compare results from

these two methods. Unfortunately, due to a technical issue associated with a commercial check valve on the MS-SID, the short-term experiments in Urania Interface were not successful. The short- and long-term experiments for the 3000m depth, however, were successful, and comparisons indicated that the phagotrophy estimators resulting from both approaches were in close agreement (Table 1). At 3000m we observed elevated values – compared to the lower mesopelagic layers - in prokaryotic daily turnover rate to $8.8 \pm 2.5\%$ with the short-term approach, and $9.8 \pm 1.7\%$ with the long-term approach. The results of the long-term study for the Urania interface indicated a turnover rate of $19.9 \pm 6.6\%$ at this depth where prokaryotic abundances are even higher than in the photic zone at 40m.

4. DISCUSSION

This study presents the first investigation of phagotrophy where the grazing impact of protists was measured using methods that allowed for *in situ* incubations, and included a depth transect in the Eastern Mediterranean Sea that extended from 40 m to 3540 m depths, where a halocline - about 1.5 meters thick - exists between normal seawater and the brine of Urania basin. Conducting such experiments in the deep sea is challenging for a number of reasons. Primarily, replication of entire experiments at each depth, although desirable, is difficult to achieve, due to wire time limitations (individual experiments required 4-16 hours of wire time). Although we were not able to perform biological replicates due to these constraints, three replicate counts were performed for each time point and depth (technical replicates) from which the standard deviation of our estimations was derived. Biological replication of experiments for selected depths in the future, will support more robust interpretations of the impacts of grazing. Consequently, we focus interpretations primarily on general trends observed, and on the contrasting results for different water masses (e.g lower mesopelagic vs interface layer).

4.1 *In situ abundances of prokaryotic and protist populations*

This is the first time that total prokaryotic and protistan abundances are reported using in situ fixed samples for euphotic, mesopelagic, and bathypelagic water masses at a single location. Concentrations of total prokaryotes and total protists (Figure 4) are within the ranges reported from other locations – sampled with hydrocasting operations – at depths in the Eastern Mediterranean Sea down to 2000 m, including the Aegean Sea, Ionian Sea, Cretan Sea and Levantine Basin (e.g Christaki *et al.*, 1999; Tanaka *et al.*, 2007; Yokokawa *et al.*, 2010). Determination of the vertical size distribution of all microorganisms was outside the scope of this study but our estimation, based on comparative microscopic observations of our samples, suggests that there is also a depth-dependent decline in size for heterotrophic flagellates and prokaryotes from the euphotic zone to the mesopelagic. Data from previous studies confirm this depth-dependent decline in both abundance and size of protists and prokaryotes (Hideki *et al.*, 2007; Tanaka and Rassoulzadegan, 2002).

The prokaryotic abundance within the Urania interface is in agreement with values found in previous studies (Borin *et al.*, 2009), while our observations for the deep-sea oxic water at 3000 m indicate elevated cell counts compared to those reported previously for the deep water mass of the same site (Borin *et al.*, 2009) and by us at the mesopelagic layers. Undisturbed bathypelagic water masses typically have fairly constant prokaryotic abundances (Tanaka and Rassoulzadegan, 2004) probably due to the fact that they are hardly (if at all) influenced by seasonality, and physicochemical characteristics of those water masses (mainly temperature, nutrient concentrations and dissolved/particulate organic carbon) are fairly stable. Since our sampling site is located above a submarine mud volcano in Urania basin with occasional upward expulsion of brine sediments caused by extreme gas pressure in the mud fluids (Hübner *et al.*,

2003), we suspect that a relatively recent eruption incident may have occurred, creating a perturbation that led to introduction of nutrients into the bathypelagic water column. Previously, Urania basin was observed to have a secondary halocline at ~3601 m depth (Borin et al. 2009). The fact that we did not observe this secondary halocline during this sampling event supports the notion of a recent disturbance, as well as the presence of fresh mud in the southeast extremity of the horse-shoe shaped Urania Basin, which is more than 5km away from volcano, located in the northwest extremity (Yakimov, personal communication).

4.2 Estimators of phagotrophy

Tracer techniques that follow labeled prey surrogates into the food vacuoles of predators are considered to be relatively robust in providing specific ingestion rates for different taxonomic classes of protists in the environment (Jürgens and Massana, 2008). Sources of potential artifacts include egestion of vacuole contents during the addition of fixatives (Pace and Baliff, 1987), prey selectivity (e.g. Landry *et al.*, 1991) and the time required for obtaining reliable counts of ingested FLPs, particularly at low grazer densities (McManus, 1991). To account for prey selectivity, we initially intended to prepare concentrated samples of the indigenous prokaryotic population from each of the studied depths. Due to time and technical constraints, such an approach was feasible only for one depth, the interface layer at 3540m. We prepared concentrated indigenous prokaryotes from this depth because the prokaryotic population there has unique characteristics due to the unique chemistry of the Urania basin halocline. For the rest of the experiments at 40, 200, 500, 950, and 3000m, a cultivable fraction of the indigenous population from the normal Mediterranean salinity seawater at 3000m was used. The use of prey prepared from 3000m water samples may have introduced a bias in the experiments conducted at 40, 200, 500 and 950m, particularly for the shallowest experiments where indigenous microbiota are

likely most distinct from those at 3000m. To minimize potential artifacts we adjusted the starvation time of the enrichment cultures in order to produce prey in the size range of the indigenous populations at each depth based on previous observations of filtered water samples.

An alternative approach for understanding protist grazing impacts for oligotrophic systems with low grazer concentrations is to follow the disappearance of added FLPs in incubations of 12– 48 hours (Marrasé *et al.*, 1992). These long-term experiments are less labor-intensive, but can only provide an estimate of phagotrophy for the whole protistan community (Jürgens and Massana, 2008). There are only a few studies directly comparing the two approaches. It has been shown, in an oligotrophic coastal system that phagotrophy estimators from long term incubation experiments were highly correlated to results from short term experiments (Unrein *et al.* 2007). Thus long term incubations are also suitable for estimating the impact of the entire protistan community. Our results of both short- and long-term grazing studies conducted at 3000m support that finding.

HNF were the main grazers observed at all depths (with the exception of *Urania* interface, where only the community phagotrophy indicators were calculated). In the euphotic zone (40 m water depth) the phagotrophic impact of pigmented nanoflagellates (PNF) was found to be less significant compared to that of HNF. This is in contrast to the findings of other studies in marine systems where the contribution of mixotrophic flagellates to bacterivory was large (e.g. Arenovski *et al.*, 1995; Unrein *et al.*, 2007). Although abundance of PNF was high (612 ± 94 cells mL^{-1}), the specific grazing rate for PNF was very low (0.31 bacteria $\text{PNF}^{-1} \text{ h}^{-1}$), resulting to a small contribution in the total grazing (Table 1).

Ciliates are known at other locations to be important grazers (e.g. Sherr *et al.*, 1989; Sherr and Sherr, 1987). In contrast, we did not observe abundant ciliates at any depth in our studies. Due to the low abundance of ciliates, we could only make a rough estimation of ciliate grazing

rates in the euphotic zone (~ 9 bacteria ciliate⁻¹ h⁻¹). This rate is among the lowest values in previous estimates for marine water columns (Sherr and Sherr, 1987). Accordingly, for the deeper water masses where the abundance of ciliates was even lower (and in some cases couldn't be determined with the volumes of water we studied), the grazing impact of ciliates appeared to be negligible. The Urania interface could be a possible exception to this observation, where slightly higher numbers of ciliates were observed, however we do not have group-specific grazing data for this depth, and therefore, we cannot make estimations. Our data for the oxic water column support previous findings from Unrein et al. (2007) that ciliates seem to have a minor effect on the top-down control of bacterioplankton in an oligotrophic coastal station in the northwest Mediterranean Sea, which is in contrast to other coastal systems (e.g salt marsh embayment on Georgia coast US) (Sherr and Sherr, 1987). These results reinforce the idea that flagellates are the main grazers on bacteria in oligotrophic marine environments. Although the impact of protistan phagotrophy upon the prokaryotic communities in the lower mesopelagic seems low in terms of daily numbers of prokaryotes removed, more in situ studies are required to estimate the growth rates of the natural prokaryotic populations. If prokaryotic growth rates are low, then our observed impact may be significant for those populations. In any case, it seems that HNF have evolved adaptations to efficiently capture bacterial cells from a relatively dilute environment (Jürgens and Massana, 2008), making them a very important group in carbon recycling in the global ocean.

Experimentally measurements of phagotrophy in the mesopelagic realm has only been performed once before in the East Sea, Korea (Cho *et al.*, 2000). The results are not directly comparable, due to the substantial temperature difference between the sampling sites and the fact that the experiments from the previous study were performed on board the ship. However, HNF

were the main grazers in both studies and the depth-dependent decline in prokaryotic abundance, HNF abundance and HNF grazing rate showed a similar trend.

At 3000m the elevated concentration of prokaryotes and HNF coincide with high (compared to the lower mesopelagic depths) phagotrophic impact of the protistan community (Figure 5). It seems that the high abundance of prokaryotes stimulated an increase in abundance of HNF and in turn, their grazing activity, reaching a grazing effect similar to that observed for the upper mesopelagic (200m). High abundance and activity of protists was also recorded for the Urania interface. Studies of oxycline layers of the Black and Baltic Seas have provided indirect evidence for high bacterivory through accumulation of ciliate membrane sterols (Wakeham *et al.*, 2007) and size-fraction shifts of chemoautotrophically fixed ^{13}C (Glaubitx *et al.*, 2008). In contrast, low impact of protist grazing was observed in the Cariaco Basin, but shifts in major prokaryotic phylogenetic groups could be detected upon removal of grazers, indicative of selective grazing (Lin *et al.*, 2007). Recently, Anderson *et al.* (2012) experimentally measured protist bacterivory for oxic-anoxic interfaces of the Baltic Sea using an approach based on the disappearance of FLPs (Marrasé *et al.*, 1992) during on-board incubations. This provided evidence that 50 to 100% of prokaryotic standing stocks were grazed daily in that habitat. The interfaces of the East Mediterranean DHABs are known to be very productive habitats in terms of prokaryotic abundance and activity (Borin *et al.*, 2009; e.g Yakimov *et al.*, 2007b). So far, only the diversity aspect of protistan communities in DHAB interfaces have been studied (Edgcomb *et al.*, 2009; Edgcomb *et al.*, 2011b; Stock *et al.*, 2012). This is study an in situ estimation of heterotrophic food-web dynamics of protists in deep-sea oxyclines/chemoclines was attempted. The phagotrophic impact on the prokaryotic community in the Urania interface is significant, with the removed percentage of the prokaryotic stock being much higher than the meso- and bathypelagic and reaching similar levels to that of the euphotic zone. Future endeavors will focus

on identifying the dominant or/and most efficient phagotrophic protistan groups in this habitat, as well as determining whether protist grazing exerts control on prokaryotic growth, and potentially on the vertical distribution of specific key prokaryotic groups that dominate oxic/anoxic interfaces. and the biochemical processes, as has been demonstrated in Baltic oxyclines (Anderson *et al.*, 2013).

5. CONCLUSIONS

This is the first study that assesses the impact of protistan phagotrophy on bacterial abundance in the mesopelagic and bathypelagic realm using *in situ* incubation experiments. Protistan grazing showed a depth-dependent decline from the euphotic zone to the mesopelagial. Although bacterial and protistan numbers are much lower in the mesopelagic layers than near the surface, phagotrophic activity probably provides a new source of labile organic compounds to bathypelagic waters.

Redoxclines are known hotspots for prokaryotic abundances and activities. In the deep sea, they likely serve as important sources of fresh labile organic matter. Our findings, together with the aforementioned previous studies, suggest that chemoclines and deep water masses where new introductions of nutrients occur, support high protist phagotrophic activity and may be important habitats of deep-sea carbon cycling. Observed grazing activities appear to have a significant impact on prokaryotic populations, with important consequences for remineralization of nutrients in these deep water-column layers. The use of new technologies, such as the MS-SID used for the present study, are likely to provide estimations of *in situ* activities of both eukaryotic and prokaryotic communities with introduction of fewer sampling artifacts, and will improve our understanding of microbial food webs in habitats that are not yet well-understood.

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625 Table 1. Estimators of phagotrophy based on equations described in the text. The asterisk (*)

626 marks the data resulting from long-term experiments.

Depth (m)	Protist Group	Clearance rate (10^{-5} mL grazer ⁻¹ h ⁻¹)	Grazing rate (bacteria grazer ⁻¹ h ⁻¹)	Grazing effect (bacteria mL ⁻¹ h ⁻¹)	Daily grazing (bacteria mL ⁻¹ d ⁻¹)	Prokaryotic turnover rate (% d ⁻¹)
40	HNF	1.12	5.78	$5.79 \cdot 10^3$	$1.39 \cdot 10^5$	26.95
	PNF	0.03	0.17	$1.06 \cdot 10^2$	$2.53 \cdot 10^3$	0.49
	Ciliates	1.76	9.05	$8.23 \cdot 10^2$	$1.98 \cdot 10^4$	3.83
200	HNF	0.94	1.04	$3.94 \cdot 10^2$	$9.45 \cdot 10^3$	8.5
500	HNF	1.16	0.63	$1.87 \cdot 10^1$	$4.49 \cdot 10^2$	0.83
950	HNF	1.32	0.75	$1.14 \cdot 10^1$	$2.74 \cdot 10^2$	0.49
3000	HNF	1.64	1.45	$3.24 \cdot 10^2$	$7.78 \cdot 10^3$	8.75
	*	1.83	1.62	$3.62 \cdot 10^2$	$8.68 \cdot 10^3$	9.77
Urania Interface	*	0.770	12.70	$1.36 \cdot 10^4$	$3.27 \cdot 10^5$	19.92

627 * data from long-term experiments

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Figure legends

Figure 1. Location of the sampling site in the East Mediterranean Sea. Modified from Yakimov et al. (2007b)

Figure 2. Microbial Sampler-Submersible Incubation Device (MS-SID). Panel (A) 3D schematic of the instrument illustrating major modular components. Panel (B) deployment of MS-SID for collection of microbial samples from Mediterranean Sea for grazing studies.

Figure 3. Salinity, oxygen and temperature profile of water column provided by the High Range CTD on the MS-SID. Panel (A) the profile of the entire water column. Panel (B) profile of the halocline above Urania basin at 3540m depth. Red and blue circles=oxygen and salinity profiles during deployment of the instrument to the halocline and while establishing ideal position of the instrument for sampling. Red and blue crosses=oxygen and salinity measurements during the time the sample for the grazing study was being drawn into the MS-SID main chamber.

Figure 4. Abundance of prokaryotes panel (A) and of protists panel (B) with depth.

Figure 5. Calculated grazing effect at each sampling depth panel (A) and the calculated daily prokaryotic turnover at each sampling depth panel (B).

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Figure 2

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